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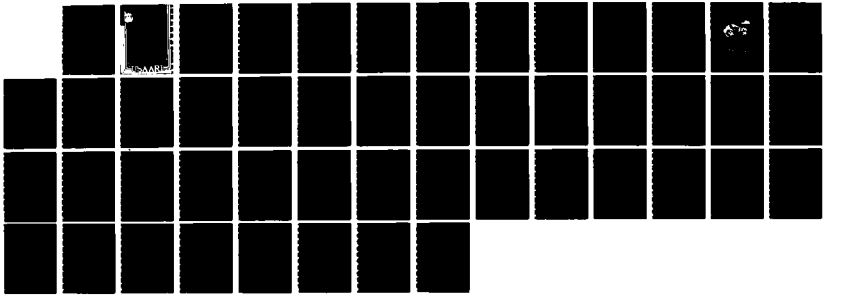

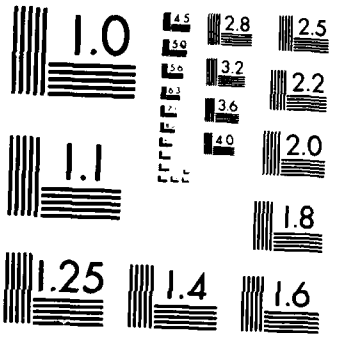

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SOME EFFECTS OF ANTICHOLINESTERASE DRUGS AND THEIR ANTIDOTES ON EXTRARETINAL PHOTORECEPTOR CELLS OF APLYSIA CALIFORNICA

By
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ABSTRACT:

Aplysia extraretinal photoreceptor (ERP) cells R_2, $LP1_1$, and VPN were used as models of phototransduction. The early steps of light transduction in Aplysia ERP cells are very similar to those proposed in the calcium scheme for vertebrate rod outer segments. The effects of clolinesterase inhibitors and their antidotes on photoresponses in Aplysia ERP cells were investigated by electrophysiological methods.

Bath application of diisopropyl fluorophosphate (DFP), a potent irreversible organophosphate-type cholinesterase inhibitor, consistently decreased the maximum amplitude of the photoresponse elicited by flashing a light on Aplysia ERP cells. DFP did not change membrane resistance, nor did it change the reversal potential for the photoresponse. Physostigmine, a reversible carbamate-type cholinesterase inhibitor, depressed both photoresponse amplitude and membrane resistance. Attenuation of photoresponse was dose-dependent with both DFP and physostigmine and was completely reversed by washing out the drugs. Physostigmine was less potent. Both drugs caused depolarization of the resting membrane potential (RMP). Pyridostigmine, another reversible carbamate-type cholinesterase inhibitor, had no effect on photoresponse or membrane resistance at five times the concentration used for physostigmine. Bath application of carbachol, which would mimic a buildup of acetylcholine (ACh) following cholinesterase inhibition, caused a persistent hyperpolarization of the RMP. Carbachol caused attenuation of both photoresponse and membrane resistance. Previous studies have shown that DFP inhibits Na, K-ATPase. Depolarization after treatment of ERP cells with 10^{-3}M ouabain, suggests that this depolarization is not caused by Na pump inhibition. The muscarinic ACh receptor antagonist atropine blocked the photoresponse attenuation caused by DFP. Atropine did not block the attenuation of photoresponse and membrane resistance caused by physostigmine and carbachol. Calcium-free, high-magnesium sea water, which blocks release of ACh and other neurotransmitters, did not block the attenuation of photoresponse cause by DFP.

The effects of DFP are reversible, and different from those of physostigmine and carbachol. Calcium-free sea water did not block DFP's effects. These results suggest that the effects of DFP on ERP cells are not due simply to a buildup of ACh at synapses subsequent to cholinesterase inhibition. Atropine's block of DFP's effects might be caused by competition for binding sites.

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The author thanks Steve Stringer and Geraldine Fields for their technical assistance, Drs. Albert W. Kirby, Jim E. Fulbrook, and Thomas H. Harding for their valuable critical comments and suggestions regarding the manuscript, and, most of all, Wanda Norton, for her skillful typing, patience, and unfailing good humor during the typing and numerous revisions of this report.

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INTRODUCTION

The possibility that organophosphate (OP) nerve agents may be used on the battlefield is an ever present threat to our combat forces. These agents cause death at high doses by respiratory depression and cardiovascular collapse. At lower doses, they cause impairment of function in many bodily systems, including the visual system. Existing antidote and pretreatment preparations also depress function in these same systems. Therefore, there is an urgent need for antidote and pretreatment preparations less likely to incapacitate the soldier. Further information about the mechanism of action of these agents on nerve cells is essential for successful design, selection, and evaluation of better drug preparations.

The accepted mechanism whereby OP nerve agents cause dysfunction and death is by inhibition of acetylcholinesterase (AChE), resulting in accumulation of acetylcholine (ACh) at receptor sites and subsequent exaggeration of the normal response to ACh. However, AChE inhibitors also have effects on cholinergic systems which do not appear to be due solely to AChE inhibition and ACh accumulation (Carlson and Dettbarn, 1983; Fossier, Baux, and Tauc, 1983), including direct effects on ion channels (Albuquerque, et al., 1984). OPs have, in addition, significant effects on noncholinergic systems, including those utilizing catecholamines, serotonin (Fernando, Hoskins and Ho, 1983), or GABA (Sivam et al., 1983) as transmitters.

The well-known effects of OPs in the visual system (pupillary constriction and spasm of accommodation) are what would be expected from AChE inhibition. AChE inhibitors cause disruption of function at other levels of the visual system (Harding, Wiley, and Kirby, 1983; Harding, Kirby, and Wiley, 1983) including the retina (Von Bredow, Bay, and Adams, 1971). These actions on the visual system may well involve other actions besides AChE inhibition. The effects of AChE inhibitors on photoreceptors have not been studied previously.

An electrophysiological investigation therefore was conducted to study the effects on photoreceptors of the irreversible organophosphate AChE inhibitor diisopropyl fluorophosphate (DFP), the reversible carbamate AChE inhibitors physostigmine and pyridostigmine, and antidote drugs such as diazepam and the cholinesterase reactivator, 2-PAM. The identified neurons of Aplysia which have been designated R_2 (Frazier et al., 1967), LPl_1 (Rayport, Ambron, and Babiarz, 1983), and VPN (Andresen and Brown, 1979) were used as a model system. These cells have well-characterized properties and can be identified in every animal. All three neurons are extraretinal photoreceptor (ERP) cells (Brown, Brodwick, and Eaton, 1977; Andresen and Brown, 1979) which produce hyperpolarizing responses to flashes of light.

Aplysia ERP cells have a photochemical transduction mechanism that is similar to the calcium scheme proposed by Yoshikami and Hagins (1971) for vertebrate rod outer segments (ROS) (Figure 1). There is considerable experimental evidence supporting both the calcium scheme and a scheme implicating cyclic GMP as an internal messenger (Hubbell and Bownds, 1979). Recently

Fatt (1982) has proposed a mechanism incorporating both schemes. Photochemical transduction in Aplysia ERP cells (Figure 2), like that in the calcium scheme for vertebrate ROS, involves the sequence of light absorption, chromophore transformation, and internal transmitter release from an intracellular calcium-sequestering organelle. Calcium release in turn provides the linkage to the output mechanism which is a change in membrane conductance (Andresen and Brown, 1982) causing hyperpolarization of the cell membrane. In Aplysia ERP cells, calcium is released from intracellular organelles called lipochondria, and activates a class of potassium channels in the cell membrane; whereas in vertebrate ROS, calcium is released from disks and inactivates the sodium "dark current" channels. Although the effect on ionic conductance is different in the two systems, the result is a hyperpolarizing photoresponse in both cases. The parallels in mechanism between the highly-specialized vertebrate ROS and the simple Aplysia ERP cells suggest that the basic phototransduction scheme is fundamentally similar in these two phylogenetically distant cases.

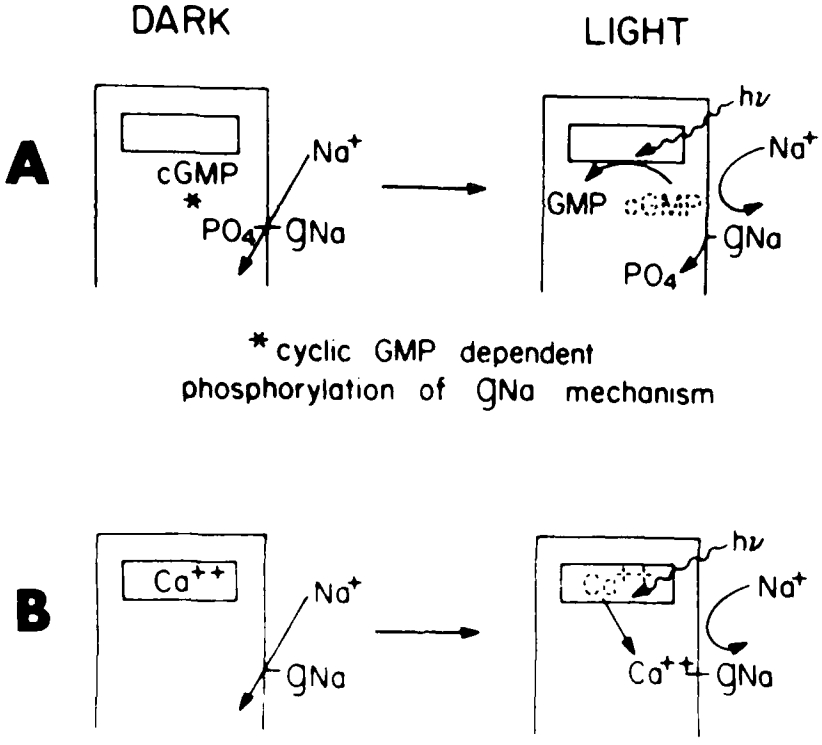

FIGURE 1. Two Proposed Schemes for Photochemical Transduction in Vertebrate Rod Outer Segments. A. Illumination causes degradation of cyclic GMP, followed by sodium channel dephosphorylation and closure. B. Calcium is released from discs By illumination, causing closure of sodium channels.

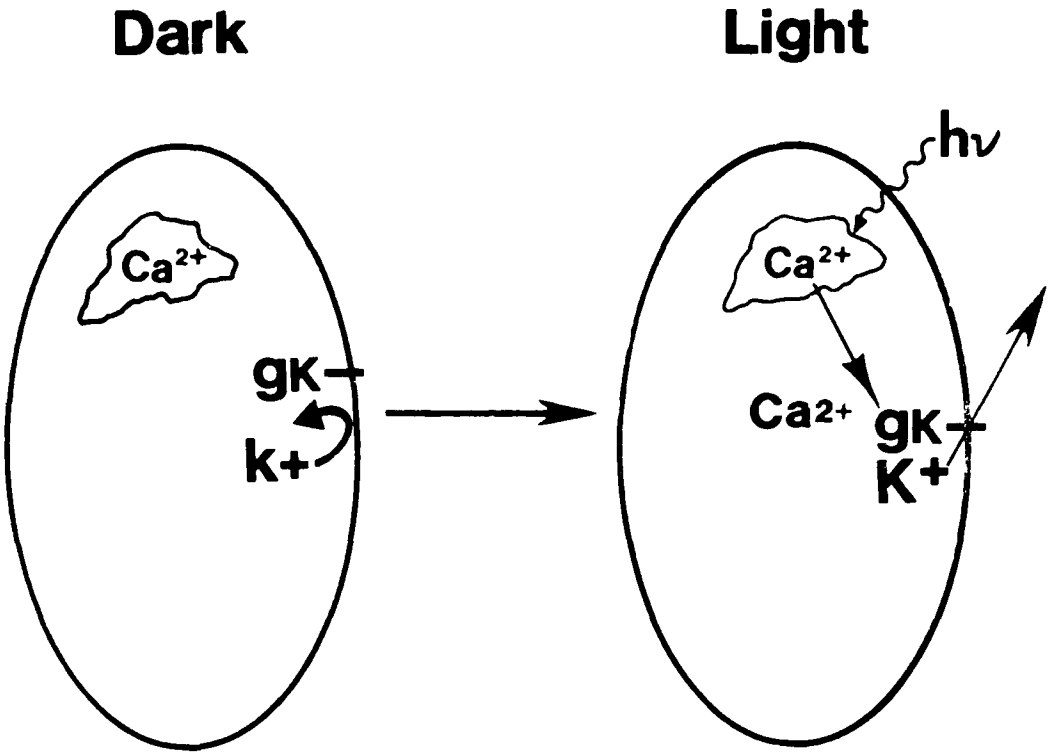

FIGURE 2. Photochemical Transduction in Aplysia Extraretinal Photoreceptors. Illumination causes release of calcium from lipochondria. Calcium ions, in turn, activate potassium channels in the cell membrane.

This preparation should yield valuable information relevant to photoreceptors in general. A further attractive feature of Aplysia ERP cells is that they are well-defined cholinergic cells (Kehoe, 1972; Carpenter, Swann, and Yarowsky, 1977; Kandel et al., 1967). Because of their large size, accessibility, and durability, and because their biophysical properties are well characterized (Marmor, 1975), these cells are particularly advantageous for electrophysiological and biochemical investigations of drugs affecting cholinergic function.

The objectives of this investigation were twofold. First, to study the effects of AChE inhibitors and of antidote drugs on the photoresponse of Aplysia ERP cells; and second, to contribute to a data base which can be used to assist in the design of more effective antidote and pretreatment drug preparations.

MATERIALS AND METHODS

Identified ERP cells in isolated ganglia of the marine mollusk Aplysia californica (Figure 3) were used in this investigation. Animals were obtained from Pacific Biomarine Laboratories*, and from Marinus, Inc.*. Data are reported from 39 animals each weighing 150-300 grams. Animals were maintained in a sea water aquarium at 15°C and fed seaweed or lettuce on alternate

*See Appendix.

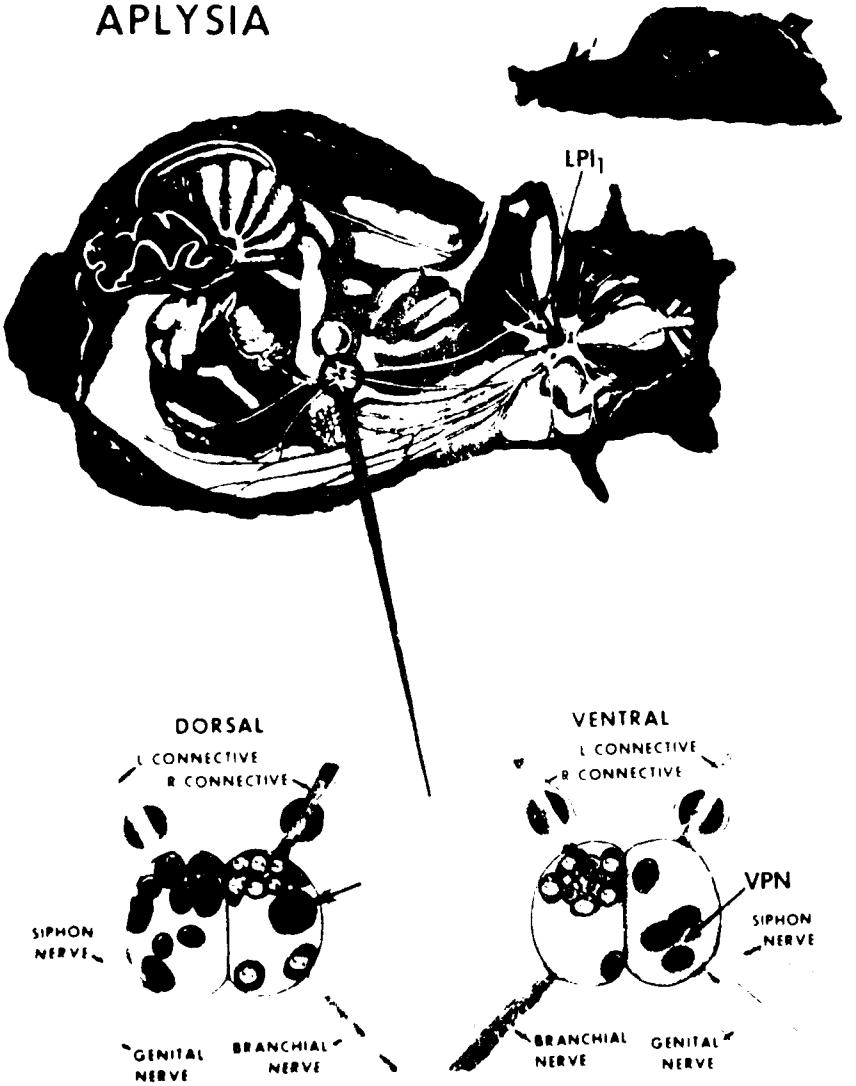

FIGURE 3. Aplysia californica with Extraretinal Photoreceptor Cell Locations Indicated by Arrows.

days. The animals were anesthetized by injecting 30 cc of 1M $MgCl_2$ into the body cavity and ganglia containing ERP cells were removed. This dissection, as well as all subsequent manipulations of the ganglia, was performed under dim red light (wavelength greater than 620 nm) in order to keep the cells dark-adapted. The ganglia were pinned in a clear acrylic chamber which allowed continuous perfusion of fluid over the ganglia during electrophysiological recording. The temperature of the fluid in the chamber was maintained at 17°C. The preparation was viewed through a stereomicroscope to allow direct visualization of the cells during impalement with microelectrodes.

The experimental apparatus (Figure 4) consisted of a standard two-electrode arrangement (Apland, 1981). Microelectrodes were 3M KCl-filled glass micropipettes with tips approximately 1 μm in diameter. The voltage-sensing electrode, which was used to monitor membrane potential, was connected via a unity-gain differential preamplifier to an oscilloscope and an oscillographic recorder. The current-injecting electrode, which was connected to a constant-current source, was used to control resting membrane potential (RMP) and to inject current pulses for measurement of membrane resistance. Once impaled, cells were usually viable for up to 48 h.

The light stimulus used to elicit photoresponses from the ERP cells consisted of a xenon arc lamp which directed light through a fiber optic bundle to the preparation, where it was focused into a 3.4 mm diameter spot with a microscope objective lens. Light was filtered to the optimum 500 nm wavelength (Andresen and Brown, 1982) using an interference filter with 50 nm band

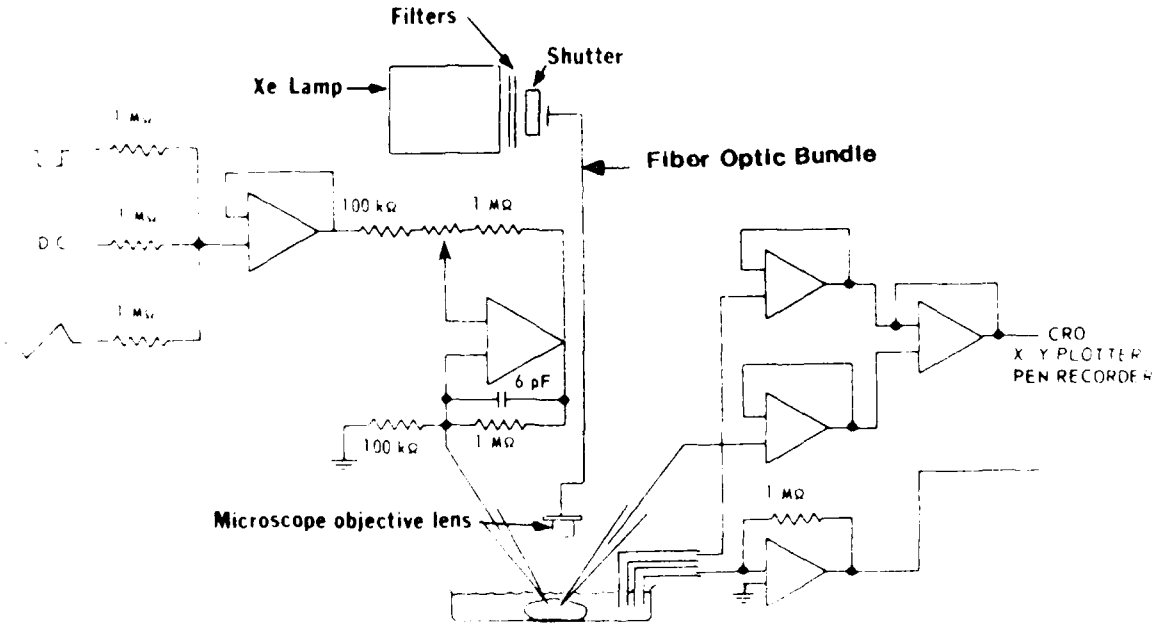

FIGURE 4. Experimental Apparatus for Electrophysiological Recording.

width, and stimulus irradiance was controlled with neutral density filters. Maximum radiant energy flux at 500 nm, measured with a radiometer at the level of the preparation, was 2.9 mW cm^{-2}. This was well above the threshold light intensity for cells R_2 (4.3×10^{-4}W cm^{-2}) and VPN (4.1×10^{-7}W cm^{-3}) determined by Andresen and Brown (1979). An electronically-driven shutter was used to control light stimulus duration, which was normally 10-30 s.

The photoresponse elicited by a light flash in Aplysia ERP cells (Figure 5) was a transient negative-going (hyperpolarizing) voltage change. In the usual experimental paradigm, several photoresponses were elicited to establish repeatability and obtain an initial response in normal artificial sea water (NASW). Next, perfusion with a test solution containing a drug such as DFP was begun. The preparation was perfused with test solution for 20 minutes to allow the RMP to stabilize and the photoresponse to recover. Another light flash was performed to elicit a test photoresponse. Then, perfusion was switched back to NASW to wash out the drug, which took from 20-30 minutes. After the RMP restabilized, a third photoresponse was elicited to demonstrate recovery. The maximum amplitudes of the photoresponses, in mV, were measured. Photoresponses normally recovered completely after washout of all drugs except ouabain. When the photoresponse did not recover, the cell always showed other signs of deterioration, such as lowered RMP and membrane resistance. If the recovery photoresponse was more than 30 percent smaller than the initial response, the cell was assumed to be deteriorating irreversibly. Data from such cells were rejected. Cell VPN spontaneously generated action potentials, so it was hyperpolarized to a holding potential below threshold to give a steady spike-free baseline before eliciting photoresponses. All cells were polarized to their original RMP or

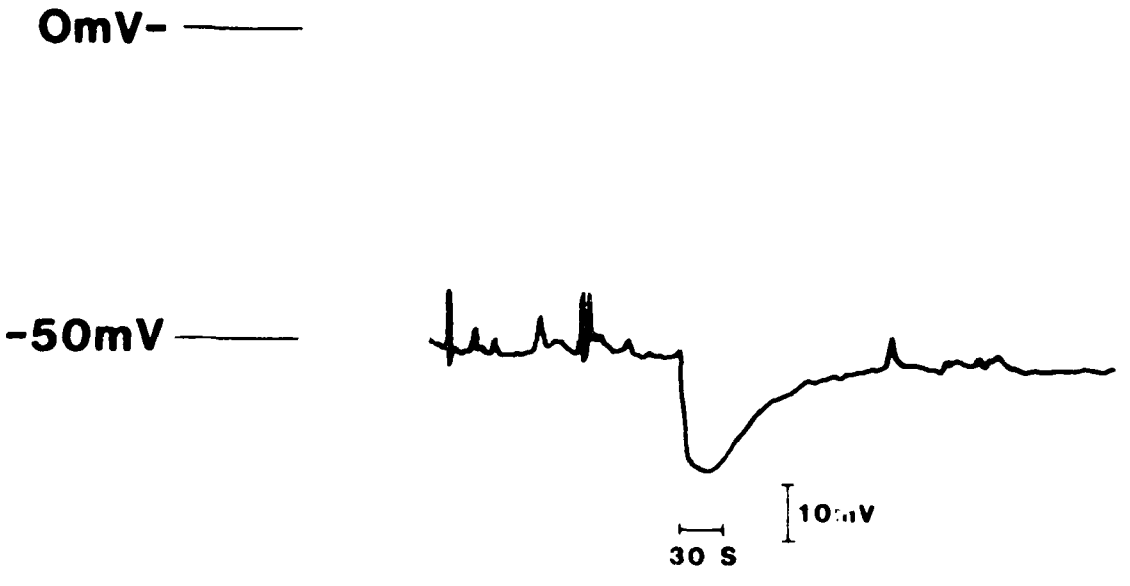

FIGURE 5. Photoresponse in Extraretinal Photoreceptor Cell VPN.

holding potential before eliciting responses, whatever RMP shift might be caused by the drugs. The maximum amplitude of the initial photoresponse, P_i, was averaged with the recovery response, P_r, to give a mean baseline photoresponse,

$$P_{\bar{x}} = \frac{P_i + P_r}{2}$$

$P_{\bar{x}}$ was subtracted from the treatment photoresponse, P_t, to give a change in photoresponse, ΔP, which was divided by $P_{\bar{x}}$ and multiplied by 100 to normalize the change for each treatment with each cell, as a percentage:

$$\%\Delta P = \frac{P_t - P_{\bar{x}}}{P_{\bar{x}}} \times 100$$

Membrane input resistance (R_M) was determined by conventional electrical methods. Small square pulses of current were injected into the cell and the resulting changes in membrane potential were measured. R_M was then calculated using Ohm's Law, $R_M = E/I$. The reversal potential (E_R) for the photoresponse, which is the potential at which the response changes polarity (from negative to positive), also was measured. This was done by eliciting photoresponses from the RMP and from a series of holding potentials more negative than the RMP. The photoresponse disappeared at E_R and became positive at holding potentials more negative than E_R.

All perfusion solutions were in NASW based on the ionic composition of Aplysia blood reported by Hayes and Pelluet (1947). The composition of NASW was: NaCl, 475 mM; KOH, 10 mM; $MgCl_2$, 20 mM; $MgSO_4$, 30 mM; $CaCl_2$, 10 mM; HEPES buffer, 10 mM; and sufficient HCl to adjust the pH to 7.7. Calcium-free sea water was prepared by substituting 100 mM $MgCl_2$ for the $CaCl_2$. Atropine, d-tubocurarine (curare), carbamylcholine chloride (carbachol), and physostigmine sulfate were obtained from Sigma Chemical Company*, diazepam from Roche Laboratories*, and DFP from Calbiochem Behring*, K & K Laboratories*, and Sigma Chemical Company*. Pyridostigmine bromide was obtained from the US Army Medical Research Institute of Chemical Defense. Pralidoxime chloride, USP (2-PAM) was a gift from Ayerst Laboratories*. Physostigmine sulfate was prepared fresh before use due to its instability in solution. Stock solutions of the other drugs were prepared in NASW. DFP was diluted to a 5×10^{-2}M stock solution in NASW and stored frozen in 4 ml aliquots until needed. The drug concentrations used were within a reasonable range for this and similar preparations (Fossier, Baux, and Tauc, 1983; Apland, 1981; Kuba, et al., 1974).

RESULTS

Basic Light Response

A typical photoresponse in extraretinal photoreceptor cell VPN is shown in Figure 5. The latency of the response is about 0.5 s. The light remained on for the 30 s indicated in the figure. The response is negative-going

(hyperpolarizing), with a maximum amplitude of about 20 mV from the RMP of -50 mV. The mean amplitudes, ± S.D., of photoresponses for the three ERP cells, in mV, were: R_2, 4.5 ± 2.1 (n=16); LPl_1, 5.9 ± 2.2 (n=4); VPN, 14 ± 5.6 (n=19).

Effects of DFP on the Photoresponse

The photoresponse was attenuated consistently by perfusion of the cells with DFP. The photoresponse shown in Figure 6 is attenuated 37 percent during perfusion of cell VPN with NASW containing 10^{-3}M DFP. Note that the photo-

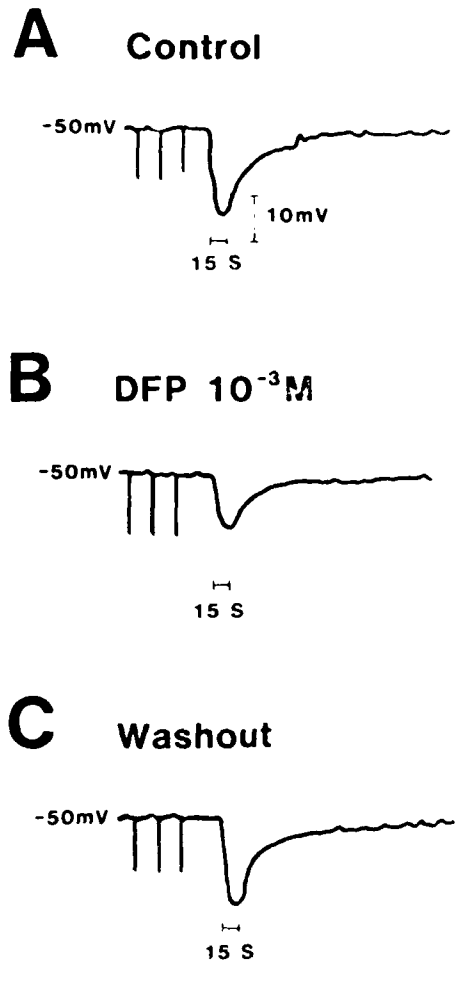

FIGURE 6. Effects of DFP on Photoresponse and Membrane Resistance. A. Control response. The three hyperpolarizing (downward-going) voltage deflections before the photoresponse were responses to (1 nA) current pulses. Their amplitude indicated membrane resistance. B. The amplitude of the photoresponse was attenuated by 37 percent in the presence of DFP, but membrane resistance was not decreased. C. Recovery of response after washout of DFP.

response completely recovers following washout of DFP, despite its being an irreversible AChE inhibitor (Filbert, 1984). An equivalent dose of DFP caused approximately equivalent inhibition of photoresponse in all three ERP cells. For example, the inhibition of photoresponse for a dose of 2×10^{-3}M DFP (mean percent inhibition ±S.D.) was: R_2, 46.2 ± 18.6 (n=13); LPl_1, 51.8 ± 17.4 (n=4); VPN, 41.8 ± 17.4 (n=24). Constant-current pulses were injected into the cell shown in Figure 6, to measure R_M. Pulses were stopped before eliciting the photoresponses, to ensure that the resulting voltage deflections would not distort the photoresponse waveforms. DFP did not reduce R_M. These results suggest that the response attenuation, and the depolarization noted in Table 2, are not due to a generalized nonspecific resistance decrease which would shunt membrane current. Therefore, a more specific effect on the phototransduction mechanism is suggested. The effects of DFP and other treatments on both photoresponse and membrane resistance are summarized in Table 1.

Effects of DFP on Resting Membrane Potential

Application of DFP typically caused the cell membrane to depolarize slowly by 5-10 mV and then stabilize over a period of 10-20 minutes (39 of 40 measurements for all cells at 2×10^{-3}M DFP). A representative experiment is shown in Figure 7. Trace A shows a VPN cell which initially was perfused with normal artificial sea water (NASW). A photoresponse was obtained, and then perfusion with 2×10^{-3}M DFP in NASW was started (left arrow). The cell membrane depolarized by about 7 mV and stabilized after 15 min perfusion with DFP sea water. The cell was repolarized to its resting potential by current injection (right arrow) before a second photoresponse was elicited. The effects of DFP and other treatments on RMP are summarized in Table 2.

A possible explanation for the depolarization caused by DFP is that it inhibits the electrogenic sodium pump, which is known to make a significant contribution to the RMP in cells R_2 and LPl_1 (Carpenter and Alving, 1968). Previous reports (e.g., Jovic, et al., 1971) have shown that OPs inhibit the sodium, potassium-ATPase. A test for inhibition of the sodium pump by DFP is to treat the cells with 10^{-3}M ouabain, which completely and irreversibly blocks the pump, causing the cell membrane to depolarize, and then to reset the cell to the original RMP by injection of current. When the cell subsequently is perfused with DFP, if it depolarizes as usual from set point, the depolarization cannot have been caused by inhibition of the sodium pump. Such an experiment is shown in Figure 7B. The cell was perfused with sea water containing 10^{-3}M ouabain to block the sodium pump and the membrane potential was reset to the original RMP by current injection (not shown). A photoresponse was elicited in ouabain sea water at the beginning of trace B, and then perfusion with DFP in ouabain sea water was begun at the left arrow. The cell membrane depolarized as it had in NASW (trace A). The depolarization in trace B could not have been caused by inhibition of the (already inhibited) sodium pump. The cell membrane was repolarized to the original set point by current injection (right arrow) and a second photoresponse was elicited. The depolarization shown in trace B was somewhat less than that in trace A, perhaps because membrane resistance was reduced by perfusion with ouabain. Note that the attenuation of photoresponse by DFP was

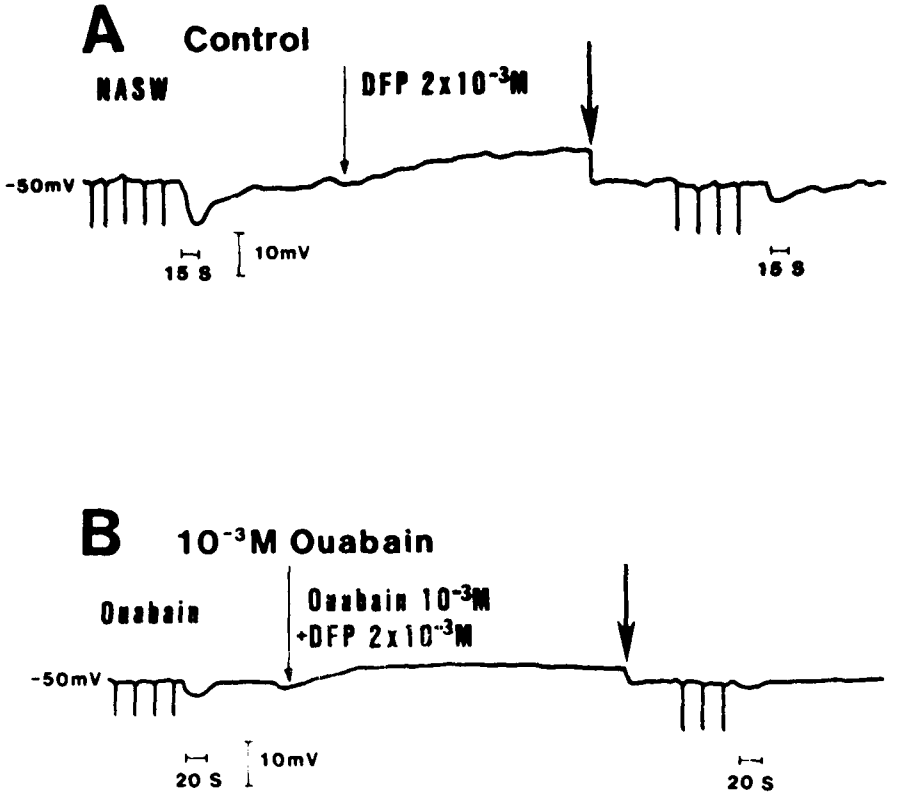

FIGURE 7. Effects of DFP and Ouabain on Resting Membrane Potential and Photoresponse. A. Control record. The cell membrane depolarized after application of DFP (left arrow). Current was injected (right arrow) to repolarize the membrane. B. Record obtained during perfusion of the cell with ouabain, with the sodium pump fully innibited. Perfusion with DFP in ouabain sea water was begun at the left arrow, and the cell membrane depolarized as it had in trace A. Current was injected (right arrow) to repolarize the membrane.

about 50 percent in both NASW and in ouabain sea water. The cell membrane depolarized when exposed to DFP in ouabain sea water in three of four cells studied. These data do not support the hypothesis that the depolarization of membrane potential caused by DFP results from inhibition of the sodium pump.

Effects of DFP on the Reversal Potential

The fact that the membrane potential depolarizes in the presence of DFP suggests that an ionic redistribution may be taking place. Such a redistribution would be expected if DFP caused a nonspecific increase in conductance of all ion channels, and might be expressed as a change in reversal potential for the photoresponse. E_R for the photoresponse is normally around -80 mV

(Andresen and Brown, 1982). A reversal potential in this range is expected for a process causing increased potassium conductance, since it is close to the equilibrium potential for potassium (-83 mV in cells R_2 and LPl_1). To test for the possibility of an ionic redistribution in the presence of DFP, reversal potentials for the photoresponse were measured in a VPN cell in both NASW and in sea water containing DFP (Figure 8). E_R almost was identical under both conditions, suggesting that no significant ionic shifts had taken place. These results support the conclusion that DFP does not cause a non-specific increase in R_2, and suggest that the effect may be more specific to the phototransduction mechanism.

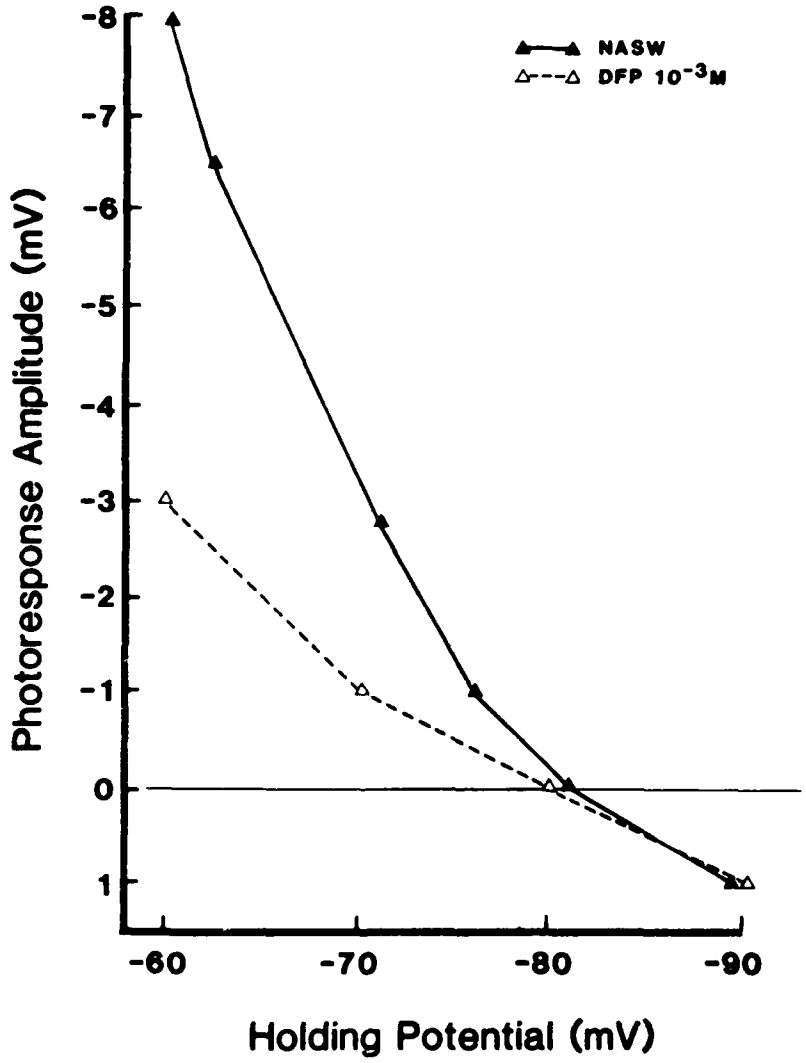

FIGURE 8. Effects of DFP on the Reversal Potential. The reversal potential for the photoresponse essentially was identical in normal sea water (81 mV) and in sea water containing 10 M DFP (80 mV).

Effects of Physostigmine and Pyridostigmine

Since physostigmine, a reversible carbamate-type AChE inhibitor, protects against the effects of organophosphates (Albuquerque et al., 1984), it was of interest to investigate the effects of this drug on ERP cells. In all six cell studies, physostigmine, at a dose of 5×10^{-3}M, attenuated the photoresponse in the same way that DFP did (Figure 9). Trace B shows that 5×10^{-3}M physostigmine attenuated the photoresponse to less than half that in NASW. Like DFP, physostigmine caused the membrane potential to depolarize slowly and then stabilize during a 10-20 minute period (not shown). However, a significant difference between DFP and physostigmine is that the carbamate also decreased membrane resistance nearly as much as it did the photoresponse (trace B). This result suggests that physostigmine may attenuate the photoresponse by a different mechanism than does DFP. Nonetheless, dose-response relationships for the two drugs (Figure 10) resemble one another. Data for

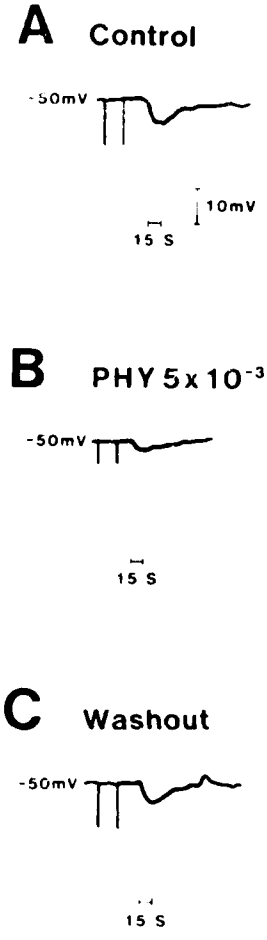

FIGURE 9. Effects of Physostigmine (PHY) on Photoresponse and Membrane Resistance. A. Control response. B. Physostigmine attenuated both photoresponse (62 percent) and membrane resistance (47 percent). C. Recovery upon washout of PHY.

all cells were pooled and averaged for each dose since response inhibition was about the same for all three ERP cells. The dose-response relationships for inhibition of photoresponse by the two drugs appeared to have similar slopes, but physostigmine was less potent.

The effects of another carbamate AChE inhibitor pyridostigmine are shown in Figure 11. This drug did not consistently attenuate either photoresponse or membrane resistance at concentrations equal to or greater than those used for physostigmine (n=4). This lot of pyridostigmine was tested for inhibition of red blood cell acetylcholinesterase and proved to be as potent as physostigmine. As frequently happened with this drug, the photoresponse and membrane resistance actually were increased by a small amount. Pyridostigmine, unlike DFP and physostigmine, did not depolarize consistently

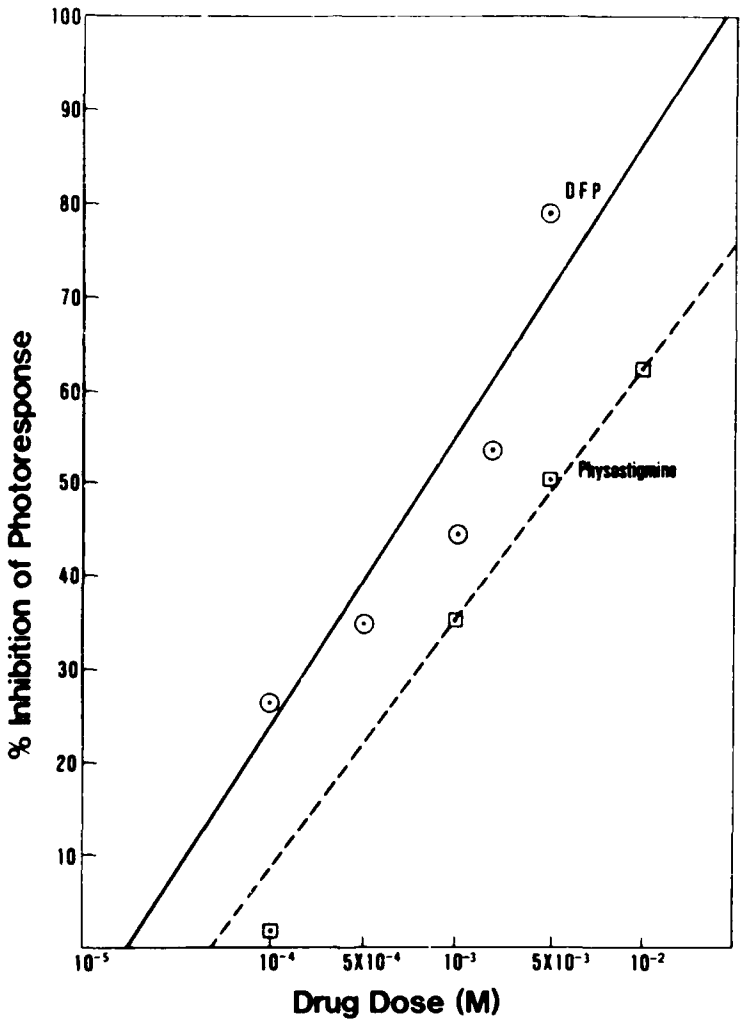

FIGURE 10. Dose-response Relationships for Attenuation of Photoresponse by DFP and Physostigmine. Data pooled and averaged.

the cell membrane. Pyridostigmine, at three different concentrations in two cells, did not prevent the attenuation of photoresponse caused by 2×10^{-3} M DFP. In Figure 12, trace B shows that DFP alone attenuated the photoresponse by about half. Pyridostigmine (trace D) at a concentration of 10^{-3} M, had no apparent effect. In trace E, DFP (2×10^{-3} M) in the presence of pyridostigmine attenuated the photoresponse as much as DFP alone (trace B). So, in this system pyridostigmine does not appear to be an effective pretreatment drug to counteract the effects of DFP.

Effects of Carbachol

Since DFP and physostigmine are AChE inhibitors, their effects might be due to buildup of ACh at synapses on ERP cells (or on other cells which, in

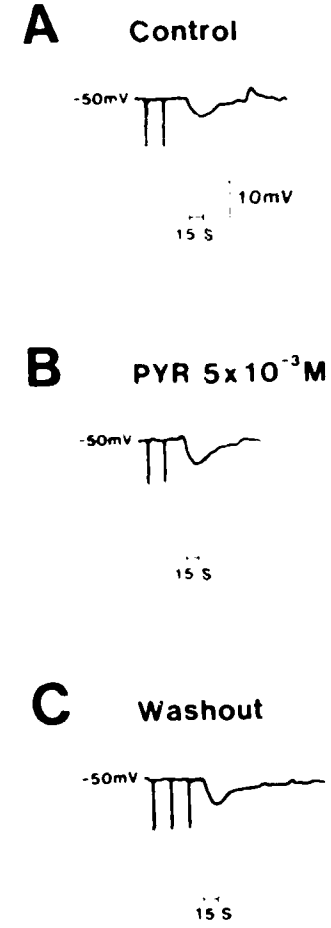

FIGURE 11. Effects of Pyridostigmine (PYR) on Photoresponse and Membrane Resistance. A. Control response. B. PYR did not attenuate either photoresponse or membrane resistance. Both were, in fact, slightly increased (by 20 percent and 9 percent, respectively). C. Response after washout of PYR.

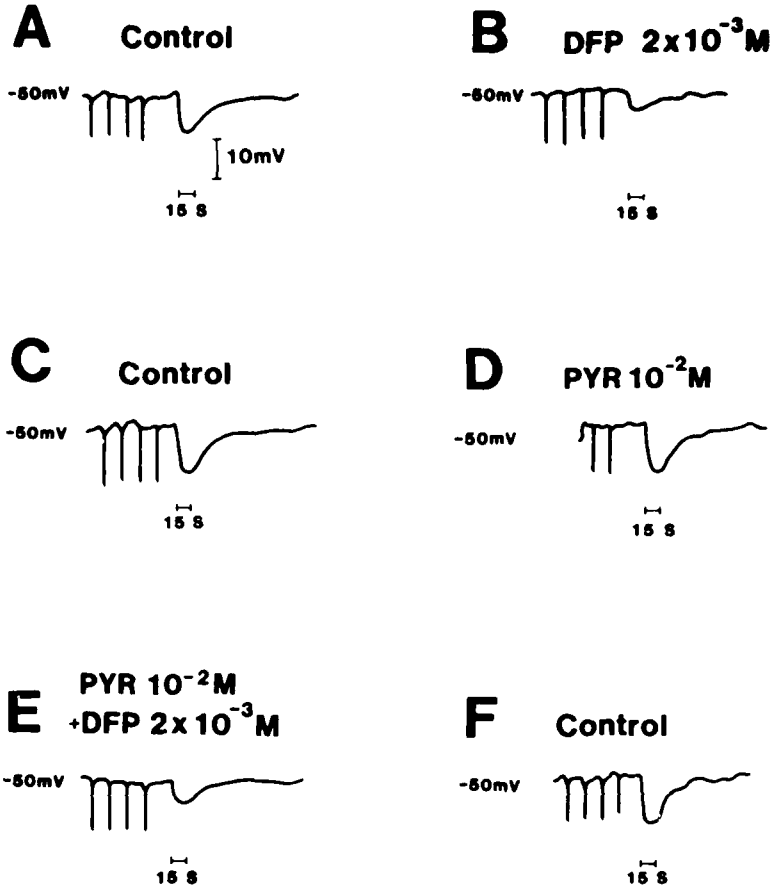

FIGURE 12. Pyridostigmine (PYR) did not Block the Attenuation of Photoresponse Caused by DFP. A. Control photorespose. B. DFP attenuated the photoresponse by 52 percent, without modifying membrane resistance. C. Control photoresponse. D. PYR did not modify photoresponse amplitude or membrane resistance. E. DFP caused an attenuation of photoresponse in the presence of PYR which was equivalent to that without PYR. F. Recovery after washout of drugs.

turn, impinge on ERP cells). Application of cholinergic drugs might be expected to mimic the effects of DFP and physostigmine. Consequently carbachol, an analog of ACh which is not hydrolyzed by AChE, was applied to VPN cells (Figure 13). Bath application of the drug (trace B) resulted in the expected resistance decrease and a hyperpolarization which peaked quickly and then settled to a plateau level between the peak hyperpolarization and the RMP. Both photoresponse and membrane resistance were attenuated. Similar results were obtained in all three cells studied. These results resemble the action of

physostigmine, but not of DFP. On the other hand, DFP and physostigmine depolarize the cell membrane, whereas carbachol causes a hyperpolarization. The data suggest that DFP and physostigmine exert their actions by some mechanism other than simple inhibition of AChE.

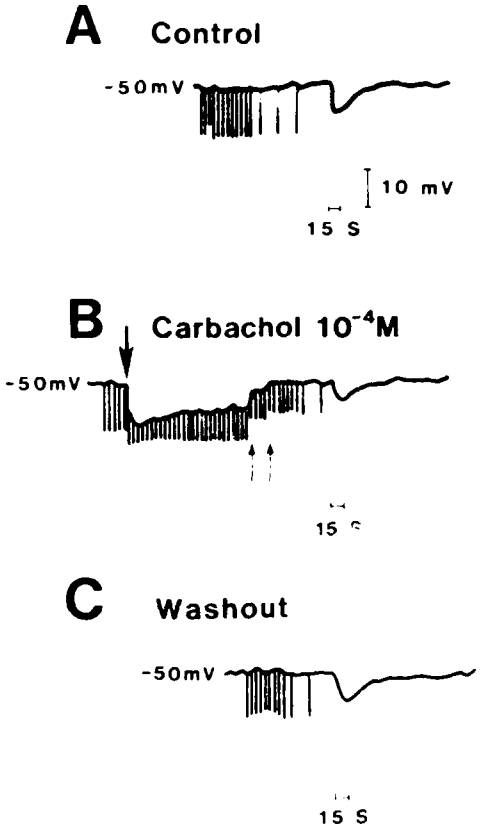

FIGURE 13. Effects of Carbachol on Resting Membrane Potential, Photoresponse, and Membrane Resistance. A. Control response. B. Bath application of carbachol begun at the arrow. Membrane potential hyperpolarized to a peak and then settled to a plateau level. Membrane potential was reset to the original resting level by current injection. Carbachol caused attenuation of both photoresponse (27 percent) and membrane resistance (28 percent). C. Recovery upon washout of carbachol.

Effects of Cholinergic Blocking Drugs

Both atropine and curare block responses associated with activation of ACh receptors. These drugs have been labeled as ACh receptor blockers even though they actually may block ionic channels associated with the receptors

(Carpenter, Swann, and Yarowsky, 1977; Slater and Carpenter, 1982). The effects of these drugs on the photoresponse in ERP cells were evaluated, as were their effects on the attenuation of photoresponse caused by DFP. Neither curare alone (eight cells, see Figure 14) nor atropine alone at concentrations less than 5×10^{-3}M (11 cells, see Figure 15) nor atropine in combination with curare (two cells, see Figure 16) caused any consistent attenuation of the photoresponse, although curare by itself occasionally accentuated the photoresponse by as much as 15 percent.

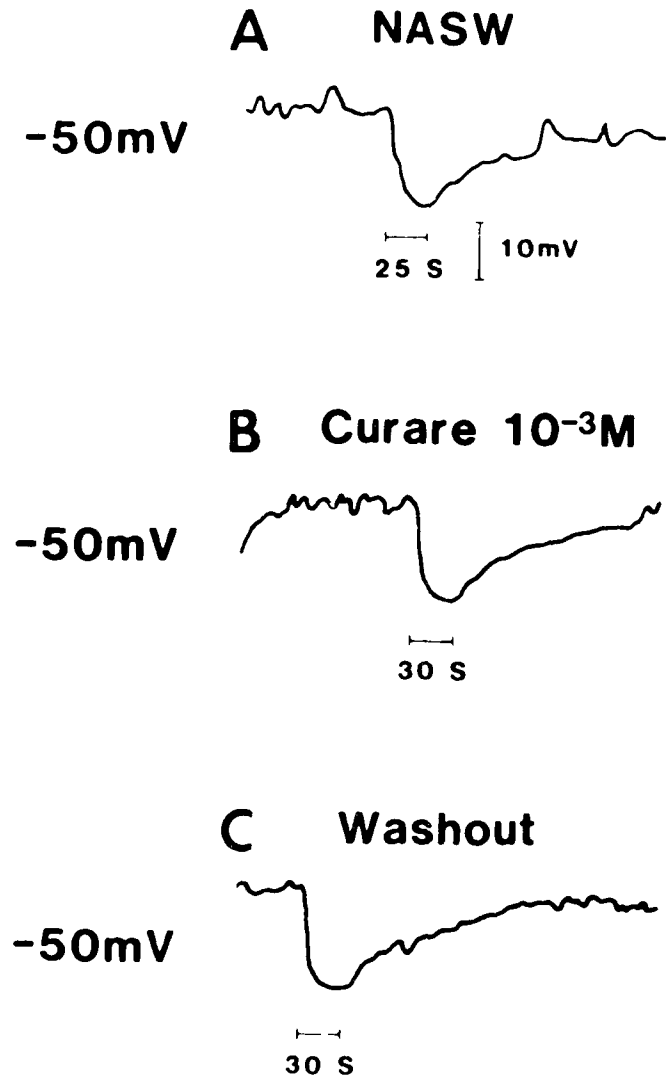

FIGURE 14. Curare Alone did not Depress the Photoresponse. A. Control photoresponse. B. Response in the presence of curare. C. Response after washout of curare.

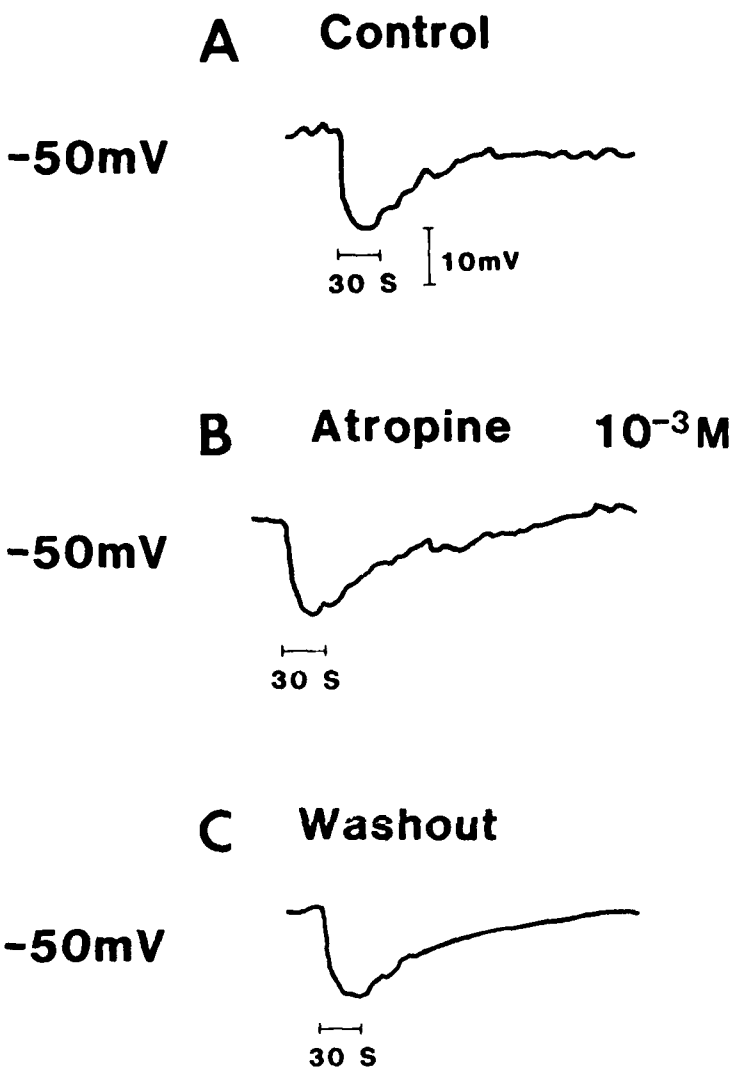

FIGURE 15. Atropine did not Affect the Photoresponse. A. Control photoresponse. B. Response in the presence of atropine. C. Response of washout of atropine.

Curare, in all three cells studied, also failed to prevent the attenuation of photoresponse caused by DFP when perfused before and then during the application of DFP (Figure 17). DFP alone caused a 49 percent attenuation of the response (trace B). Trace C represents a control response following washout. The response in trace E, in the presence of both curare and DFP, was nearly the same as in trace B. So, curare did not provide any significant protection from the action of DFP.

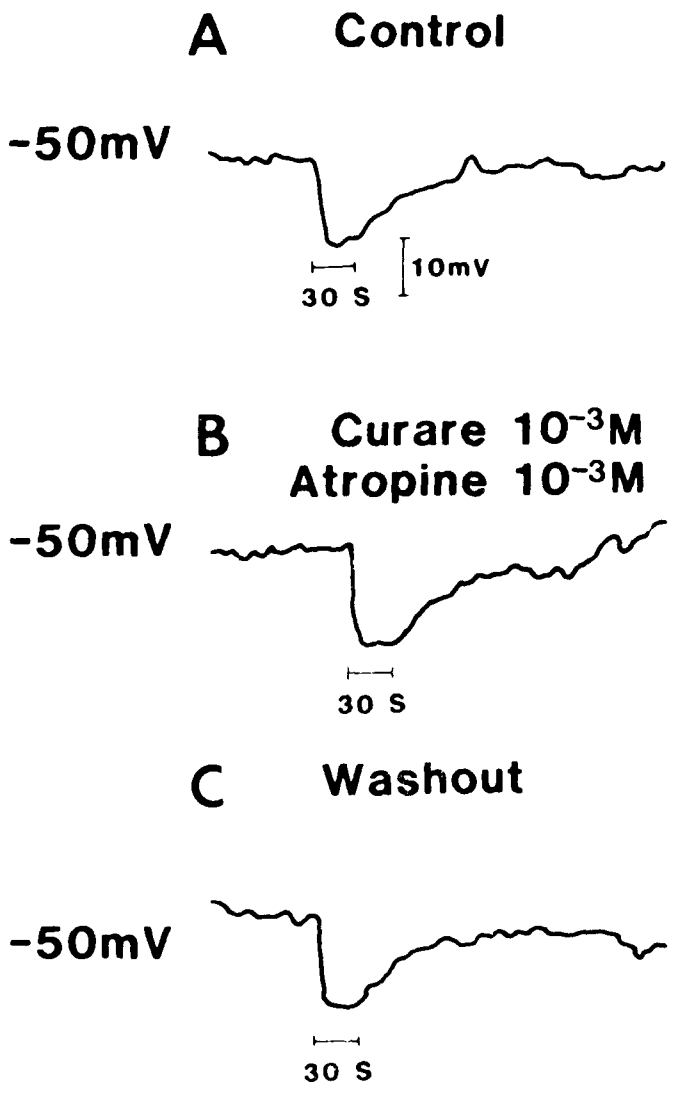

FIGURE 16. Curare and Atropine in Combination did not Affect the Photoresponse. A. Control response. B. Response in the presence of curare and atropine. C. Response after washout of drugs.

Atropine, however, decreased the attenuation of photoresponse caused by DFP in all six cells studied (Figure 18). As seen in trace B, DFP reduced the photoresponse by about one-half. But in trace E, atropine, when perfused with DFP in equimolar amounts, decreased DFP's attenuation of the response to only 16 percent from 48 percent. The response was recovered after washout. When atropine and curare were combined and perfused with DFP (Figure 19), they decreased the photoresponse attenuation caused by DFP. Atropine was the efficacious agent in this mixture in all three cells studied. DFP caused a 70 percent attenuation of the photoresponse in trace B. The response

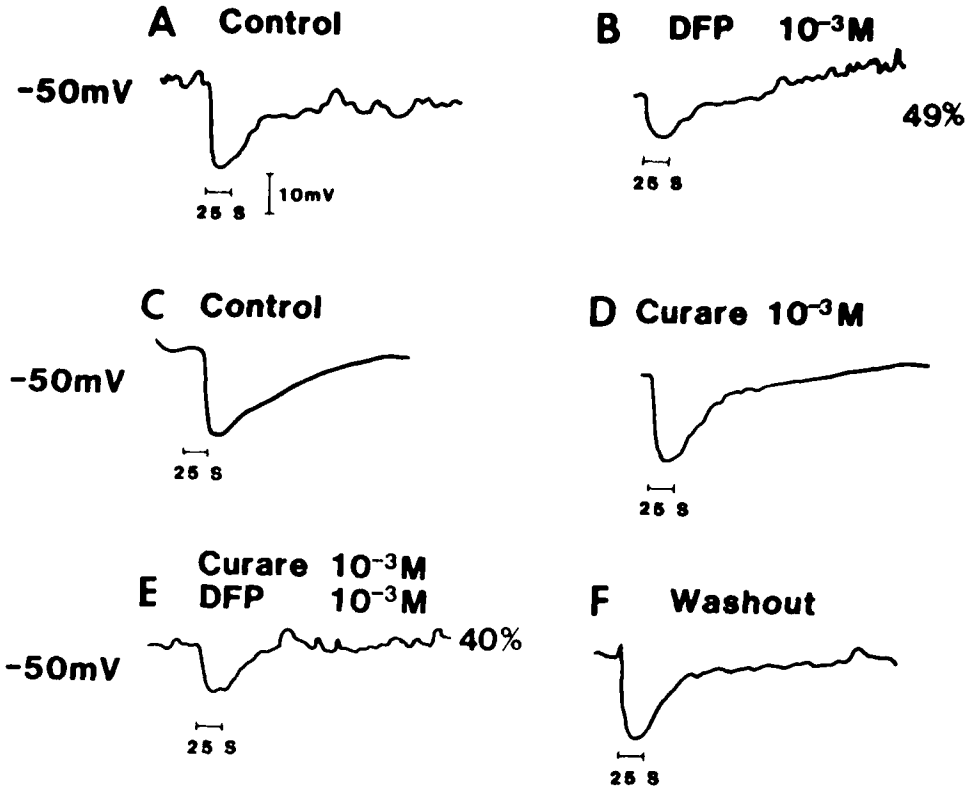

FIGURE 17. Curare did not Prevent the Attenuation of Photoresponse Caused by DFP. A. Control photoresponse. B. Response was attenuated by DFP. C. Later control response. D. Response in the presence of curare alone was not depressed. E. Response was attenuated, in the presence of DFP and curare, to about the same degree as with DFP alone. F. Response after washout of drugs.

attenuation was only half as great (35 percent) when atropine and curare were perfused with DFP (trace D). When curare was removed from the mixture leaving only atropine and DFP, shown in trace F, the response attenuation was the same as in trace D. So, it was atropine, not curare, which decreased the effect of DFP on the photoresponse. This evidence reinforces the data presented in Figure 17, which showed that curare by itself failed to protect the photoresponse from attenuation by DFP.

Atropine was not effective in blocking the effects of physostigmine in either of two cells studied (Figure 20). Physostigmine alone attenuated both photoresponse and membrane resistance by about half (trace B). Atropine was applied at the same concentration as physostigmine (5×10^{-3}M). At this higher concentration, atropine (trace D) attenuated photoresponse amplitude

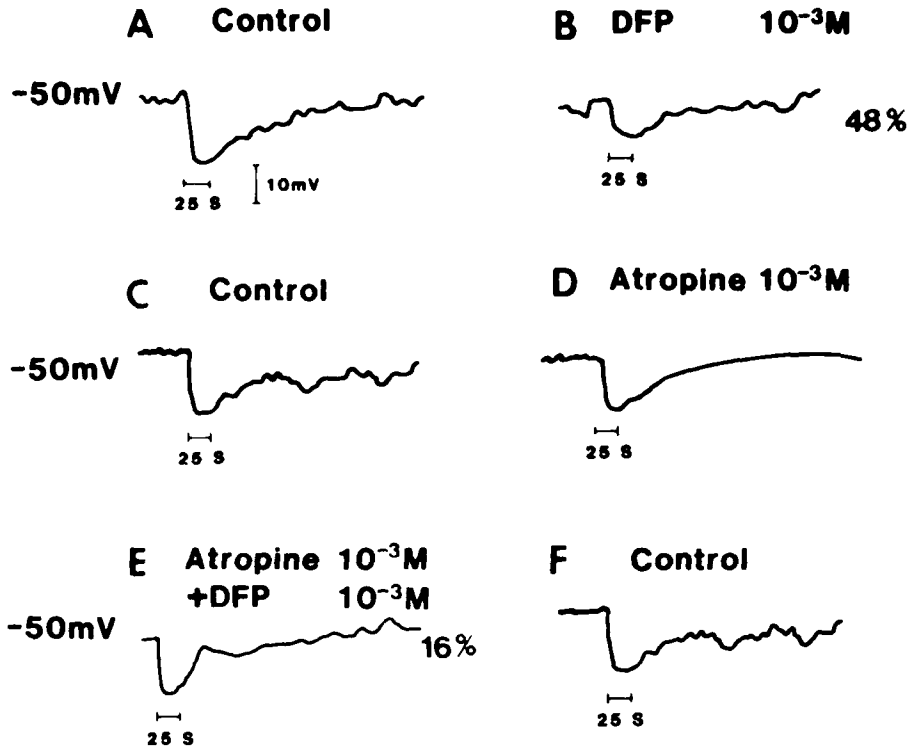

FIGURE 18. Atropine Blocked the Attenuation of Photoresponse Caused by DFP. A. Control response. B. DFP depressed photoresponse by 48 percent . C. Control response. D. Atropine alone had no significant effect. E. The response was attenuated significantly less (16 percent) in the presence of DFP plus atropine than in DFP alone. F. Recovery upon washout of drugs.

and membrane resistance about 25 percent, which was about half as much as had physostigmine. When physostigmine was added to atropine-containing sea water (trace E), membrane resistance was attenuated further to half that in atropine alone (trace D). However, photoresponse amplitude was attenuated still further, to about one-fourth that in atropine alone, suggesting a possible synergistic effect. At any rate, atropine definitely did not block the effects of physostigmine.

Atropine also was ineffective in blocking the effects of carbachol in one cell (Figure 21). The drugs, when applied alone at 10^{-4}M concentration (trace B), caused an attenuation of photoresponse of about half, and a smaller decrease in membrane resistance. Atropine alone (trace D) did not decrease photoresponse amplitude. Carbachol, when added in equimolar concentration to sea water containing atropine, reduced photoresponse and membrane resistance by half (trace E), compared to those parameters in atropine alone.

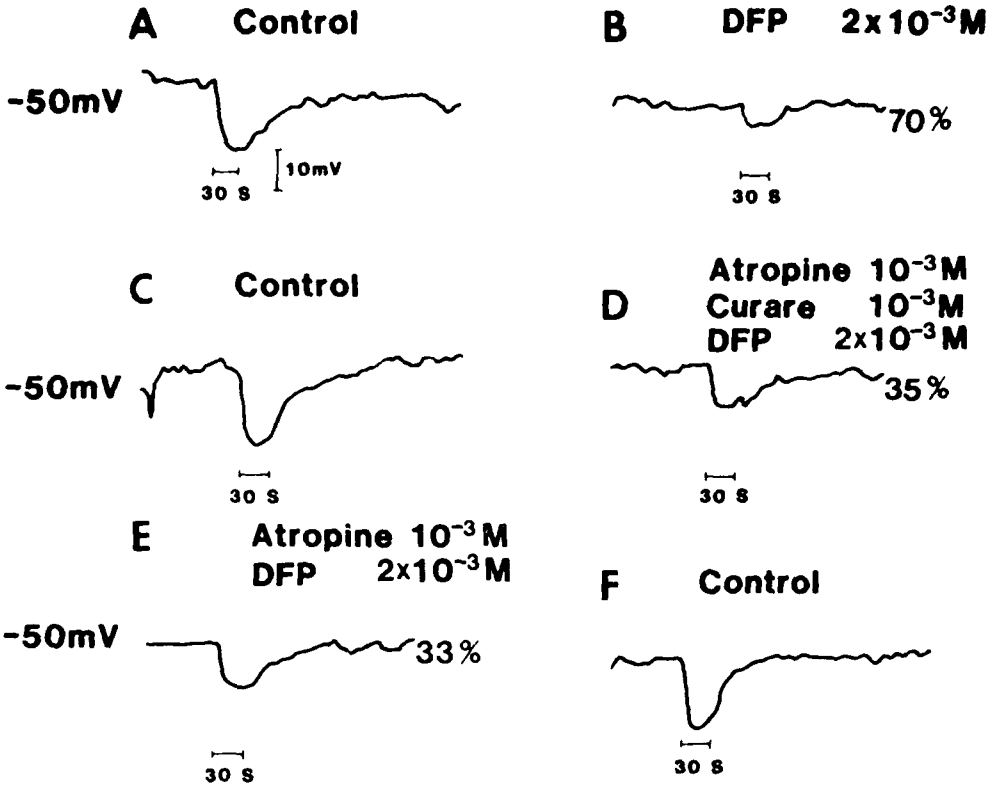

FIGURE 19. Atropine, but not Curare, Greatly Decreased the Attenuation of Photoresponse Caused by DFP. A. Control photoresponse. B. DFP greatly attenuated the response. C. Control response. D. Response attenuation was only half as great in DFP plus atropine and curare. E. Response attenuation was the same in DFP plus atropine as it was in DFP plus atropine and curare. F. Recovery after washout of drugs.

Effects of Calcium-Free Sea Water

Since DFP and physostigmine are AChE inhibitors, their ability to attenuate the photoresponse in ERP cells may be synaptically (perhaps polysynaptically mediated). In that case, perfusing the cells with calcium-free high-magnesium sea water, which blocks neurotransmitter release, should prevent accumulation of ACh or other transmitters at receptor sites, and therefore prevent the attenuation of photoresponse. The photoresponse would not be blocked completely by calcium-free sea water, even though it is a calcium-mediated process, because it depends primarily on the release of intracellular calcium. The results of such an experiment are shown in Figure 22. Both cells tested gave nearly identical results. DFP (10^{-3}M) depressed the

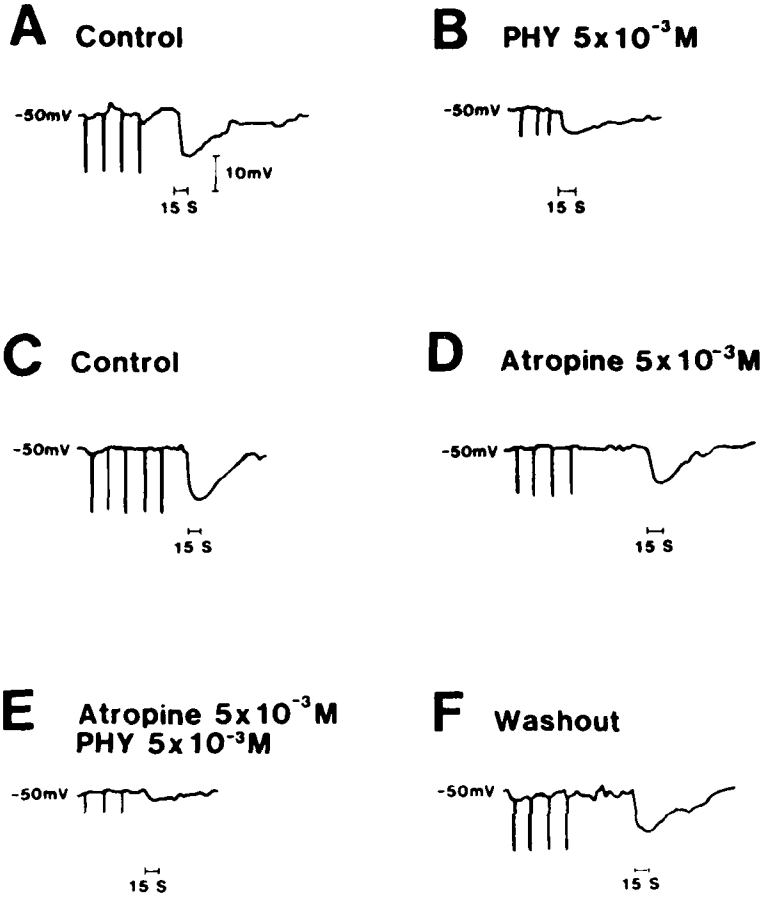

FIGURE 20. Atropine did not Block the Attenuation of Photoresponse and Membrane Resistance Caused by Physostigmine (PHY). A. Control response. B. Physostigmine attenuated both photoresponse (55 percent) and membrane resistance (53 percent). C. Control response. D. Atropine at this concentration somewhat attenuated photoresponse (26 percent) and membrane resistance (23 percent). E. DFP in the presence of atropine, attenuated both photoresponse (72 percent) and membrane resistance (46 percent), compared to the measurements in atropine alone. F. Recovery upon washout of drugs.

photoresponse by 37 percent (trace B). Both photoresponse and membrane resistance were decreased by about half in calcium-free sea water (trace D). When DFP (2×10^{-3}M) was added to calcium-free sea water (trace E), the photoresponse, but not membrane resistance, was reduced further by about half. So, blocking transmitter release did not block the attenuation of photoresponse by DFP. Calcium-free sea water also failed to prevent the actions of physostigmine on photoresponse and membrane resistance in one cell tested.

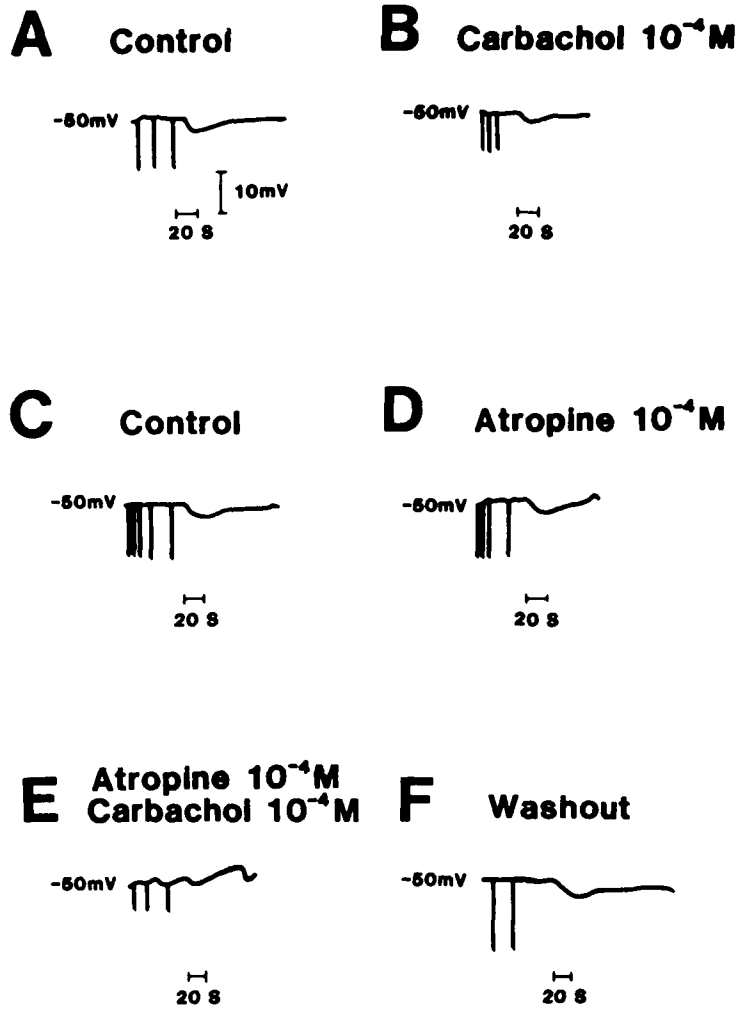

FIGURE 21. Atropine did not Block the Attenuation of Photoresponse and Membrane Resistance Caused by Carbachol. A. Control response. B. carbachol depressed both photoresponse (57 percent) and membrane resistance (31 percent). C. Control response. D. Atropine caused only a small (14 percent) decrease in photoresponse. E. carbachol, in the presence of atropine, attenuated photoresponse (50 percent) and membrane resistance (47 percent) compared to measurements in atropine alone. F. Recovery upon washout of drugs.

Dose-Dependent Effects of Various Treatments on Photoresponse, Membrane Resistance, and Resting Membrane Potential

Table 1 shows a variety of dose-dependent effects of various treatments on photoresponse and membrane resistance. Note that atropine is the only treatment which blocks the attenuation of photoresponse caused by DFP. Also, DFP is the only drug whose action is blocked by atropine. Dosages selected were those for which the most data were available.

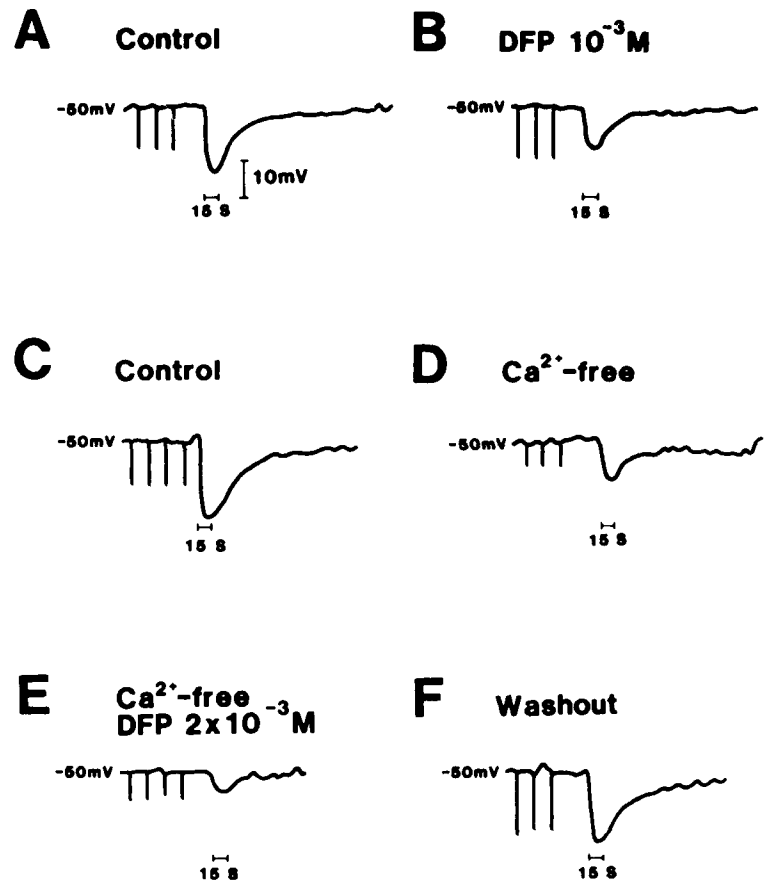

FIGURE 22. Calcium-free, High-magnesium Sea Water did not Prevent Attenuation of Photoresponse by DFP. A. Control response. B. DFP attenuated the photoresponse (37 percent), but caused a small (15 percent) increase in membrane resistance. C. Control response. D. Calcium-free sea water attenuated both photoresponse (45 percent) and membrane resistance (52 percent). E. DFP, in calcium-free sea water, attenuated the photorespons (52 percent) and slightly increased membrane resistance (7 percent) compared to measurements in calcium-free sea water. F. Recovery upon return to normal sea water without DFP.

The various drugs also had a variety of dose-dependent effects on the resting membrane potential (RMP) of the ERP cells (Table 2). The doses selected for inclusion in this table were those for which the most experimental data were available for all three ERP cells. DFP, physostigmine, and atropine consistently caused dose-dependent depolarization of up to 18 mV in all cells. Curare, alone or in combination with atropine, caused a hyperpolarization of up to 20 mV. DFP caused a depolarization when mixed with any of the above drugs.

TABLE 1

EFFECTS OF TREATMENTS ON PERCENT CHANGE IN PHOTORESPONSE (%ΔP) AND PERCENT CHANGE IN MEMBRANE RESISTANCE ($\%\Delta R_M$) IN EXTRARETINAL PHOTORECEPTOR CELLS (SELECTED DOSES)

Treatment	Dose (M)	n	Mean %ΔP ± SE	n	Mean $\%\Delta R_M$ ± SE
DFP	10^{-3}	18	-38.1 ± 5.9	6	4.9 ± 8.8
	2×10^{-3}	26	-47.6 ± 1.4	2	6.1 ± 10.5
	5×10^{-3}	5	-77.8 ± 3.2	1	-5.9
Physostigmine	5×10^{-4}	1	-60	1	-64.6
	5×10^{-3}	5	-53.1 ± 2.5	3	46.5 ± 3.8
Pyridostigmine	5×10^{-3}	3	10.1 ± 7.7	3	5.2 ± 7.3
	10^{-2}	1	-1.8	1	7.1
Carbachol	10^{-4}	3	-49.6 ± 11.7	2	-29.5 ± 1.6
Atropine	10^{-3}	5	+10 ± 4.5	2	-9.7 ± 1.5
	2×10^{-3}	2	+0.3 ± 8.9	2	-12.5 ± 8.5
	5×10^{-3}	2	-26.9 ± 10.2	2	-23.7 ± 5.8
Atropine + DFP	10^{-3} 10^{-3}	2	-16.4 ± 0.35	1	+8.9
Atropine + DFP	2×10^{-3} 2×10^{-3}	2	0 ± 12.5	2	24.8 ± 16.5
Atropine + Physostigmine	5×10^{-3} 5×10^{-3}	2	-76.1 ± 3.9	2	-37.1 ± 9.1
Atropine + Pyridostigmine	5×10^{-3} 5×10^{-3}	1	0	1	+7.5
Atropine + Carbachol	10^{-4} 10^{-4}	1	-50	1	-46.8
Calcium-free		4	-49.35 ± 5.8	3	35.4 ± 10.3
Calcium-free + DFP	2×10^{-3}	1	-52.2	1	+7.1
Ouabain	10^{-3}	2	-27.8 ± 5.6	2	-36.1 ± 5.4
Ouabain + DFP	10^{-3} 10^{-3}	2	-57.8 ± 2.2	1	-16.7
Ouabain + DFP	10^{-3} 2×10^{-3}	1	-66.7	1	+29.3
Ouabain + Physostigmine	10^{-3} 5×10^{-3}	1	-55.6	1	-33

TABLE 2

EFFECTS OF TREATMENTS ON RESTING MEMBRANE POTENTIAL (ΔRMP) IN EXTRARETINAL PHOTORECEPTOR CELLS (SELECTED DOSES)

Treatment	Dose	Direction of ΔRMP	n	Mean ΔRMP(mV)	Range (mV)
DFP	2×10^{-3}	Depolarization	40	6.9	0-17
	5×10^{-3}	Depolarization	7	8.9	2-14
Physostigmine	2×10^{-3}	Depolarization	2	4	2-6
	5×10^{-3}	Depolarization	5	13.2	5-18
Pyridostigmine	5×10^{-3}	Hyperpolarization	4	0.5	-5 to +2
Carbachol	10^{-4}	Hyperpolarization			
Peak			3	-13	-6 to -21
Plateau			3	-6.5	-3.5 to -10
Atropine	2×10^{-3}	Depolarization	2	7.5	7-8
	5×10^{-3}	Depolarization	4	10.5	7-15
Atropine + DFP	2×10^{-3} 2×10^{-3}	Hyperpolarization	2	-6.5	-6 to -7
Atropine + Physostigmine	5×10^{-3} 5×10^{-3}		2	0	-2 to +2
Atropine + Carbachol	10^{-4} 10^{-4}	Hyperpolarization			
Peak			1	-5.5	
Plateau			1	0	
Curare	10^{-4}	Hyperpolarization	8	-8.2	-3 to -14
Curare + DFP	10^{-3} 2×10^{-3}	Depolarization	5	8.6	2 to -10
Calcium-free		Depolarization	4	12.8	12 to 14
Calcium-free + DFP	10^{-3}	Depolarization	1	4	
Ouabain	10^{-3}	Depolarization	5	14.6	8 to 20
Ouabain + DFP	10^{-3} 10^{-3}	Depolarization	4	7.3	0 to 20
Ouabain + DFP	10^{-3} 2×10^{-3}	Depolarization	1	5	
Ouabain + Physostigmine	10^{-3} 5×10^{-3}	Depolarization	1	7	

DISCUSSION

The attenuation of photoresponse in ERP cells by DFP and physostigmine was very similar. The dose-response relationships for both drugs appeared nearly parallel, though physostigmine was somewhat less potent. The effects of both drugs were completely reversible upon washout. However, DFP and physostigmine were strikingly different, in that physostigmine attenuated both photoresponse and membrane resistance, whereas DFP attenuated only the photoresponse. Also, atropine blocked the effects of DFP, but not those of physostigmine. These results suggest that DFP and physostigmine may be acting by different mechanisms.

The complete reversibility of the effects of DFP on photoresponse, following washout of the drug, was an unexpected finding. Aplysia neuronal AChE is inhibited completely and irreversibly by 10^{-6}M DFP (Filbert, 1984), which is at least two orders of magnitude less than the drug concentrations used in this study. The conventional interpretation of the action of DFP on a physiological function is that the effects are due to inhibition of AChE. Enzyme inhibition would result in buildup of ACh at synapses and subsequent increased receptor activation. Receptor activation somehow may inhibit the photoresponse, perhaps by inhibiting activation of potassium channels by calcium, or by inhibiting calcium release or transport to the membrane. Atropine could depress DFP's effect by blocking receptor activation. However, if attenuation of the photoresponse were due to AChE inhibition, DFP should have an effect only the first time it is applied. Subsequent applications of DFP should have no effect since AChE already would be fully and irreversibly inhibited unless there is a sequestered store of AChE which is protected from inhibition by DFP and is released subsequently over a period of time.

Other results indicating that DFP was not acting only by AChE inhibition were obtained in the present study. Bath application of carbachol, which would mimic the effects of AChE inhibition, did not produce effects identical to those of DFP treatment. Carbachol did attenuate the photoresponse, but it also depressed membrane resistance (DFP did not) and caused hyperpolarization of the cell membrane (DFP caused depolarization). In addition, the use of calcium-free, high-magnesium sea water, which should block transmitter release and accumulation, did not change DFP's effects. Furthermore, DFP doses of 10^{-4}M to 10^{-2}M, which are 2-4 orders of magnitude greater than the dose needed to inhibit AChE completely and irreversibly, cause dose-dependent, graded attenuation of the photoresponse (Figure 10). These results suggest that the effects of DFP on the photoresponse in Aplysia ERP cells are due to some mechanism other than inhibition of AChE.

Of all the drugs tested, only atropine decreased the attenuation of photoresponse caused by DFP. Preliminary findings with the AChE reactivator, 2-PAM and diazepam, both of which have been used in the treatment of OP toxicity, indicate that these drugs have no effect on the photoresponse by themselves, and were not effective in blocking DFP's effects upon the photoresponse. The same is true of pyridostigmine, which is a proposed pretreatment drug. Curare was used since it blocks the hyperpolarizing chloride conduct-

ance increase in response to application of ACh and other neurotransmitters in Aplysia cells (Kehoe, 1972; Carpenter, Swann, and Yarowsky, 1977). The ERP cells all have hyperpolarizing responses to ACh. Since curare blocks such responses, it was thought that it might block the photoresponse. But it did not, nor did it block DFP's effect on the photoresponse. On the other hand, available evidence indicates that atropine blocks only the depolarizing sodium response to ACh (Kehoe, 1972), which is not believed to be present in ERP cells. It was used because it is one of the specific treatments for OP toxicity, and it was effective in decreasing DFP's attenuation of the photoresponse.

The photoresponse in Aplysia ERP cells is dependent upon activation of potassium channels, and the AChE inhibitors may directly inhibit the calcium-activated potassium channels which mediate the light response. Such an action would not be without precedent. Fossier, Baux, and Tauc (1983) reported that responses to iontophoretic application of both ACh and carbachol were increased after inhibition of AChE by OPs. Since carbachol is not hydrolyzed by AChE, the increase in responses could not result from AChE inhibition alone. A direct action on ion channels was inferred. Fossier, Tauc, and Baux (1983) found that oximes at high concentrations, which inhibited AChE as did OPs, also inhibited a voltage-dependent sodium conductance in Aplysia neurons. It is possible that DFP directly blocks the calcium-activated potassium channels or calcium release or transport. The fact that the reversal potential for the photoresponse does not change in the presence of DFP indicates that the drug has a specific action on a type of channel associated with the phototransduction mechanism, rather than a nonspecific action on all channels. Further evidence for this point is the fact that DFP did not change the membrane resistance of ERP cells. Depression of DFP's action by atropine, which has been considered to be a blocker of receptor-activated channels (Slater and Carpenter, 1982), suggests that atropine may be preventing the access of DFP to its sites of action.

The possibility that both DFP and physostigmine may act directly on ion channels suggested further by the work of Albuquerque et al. (1984). These authors have shown that physostigmine appears to block the ionic channel associated with the ACh receptor in the frog neuromuscular junction. The authors also demonstrated that pyridostigmine interacts with the ACh receptor as a weak agonist which can cause desensitization. A difference in mechanism of action between physostigmine and pyridostigmine also was found in the present study, since physostigmine accentuated both photoresponse and membrane resistance, and pyridostigmine did not.

Drugs also have a variety of effects on the RMP of ERP cells. The action of ACh on all the cells is hyperpolarizing, as demonstrated by bath application of carbachol (Figure 11). So DFP and physostigmine, which inhibit AChE and cause accumulation of ACh, should hyperpolarize the cells. Instead, they depolarize them. Desensitization to ACh might explain a decay of hyperpolarization after a prolonged period, but not depolarization. Curare, which blocks the hyperpolarizing chloride responses to ACh, should depolarize the cells if no other systems are active. Instead, curare hyperpolarizes the cells. Atropine, which blocks the depolarizing sodium response (though this

type of response to ACh is not thought to be present in these cells), might be expected to hyperpolarize the cells. Instead, it depolarizes them. One possibility is that DFP and similar drugs depolarize ERP cells by blocking the electrogenic sodium pump. However, the results obtained with ouabain in this study (Figure 7) do not support this mechanism.

CONCLUSIONS

1. The AChE inhibitors DFP and physostigmine consistently attenuated the photoresponse in Aplysia extraretinal photoreceptor cells in the experiments reported here. Atropine blocked the effects of DFP, but not of physostigmine. In addition, physostigmine depressed membrane resistance, whereas DFP did not. This suggests a more specific action of DFP on the photoresponse.

2. The photoresponse attenuation was reversed completely upon washout of both drugs. AChE activity would be expected to be completely and irreversibly inhibited at DFP concentrations much lower than those used in this study. Yet, graded effects on the photoresponse were obtained with several doses of DFP above the threshold for AChE inhibition. Calcium-free sea water did not block the effects of DFP and physostigmine, and the effects of these drugs and carbachol were dissimilar. These results suggest that DFP attenuates the photoresponse by a mechanism other than AChE inhibition and accumulation of ACh.

3. Several drugs which have been used in the treatment of organophosphate intoxication were tested in this study. Preliminary evidence indicates that diazepam and the AChE reactivator 2-PAM did not prevent the attenuation of photoresponse caused by DFP. The proposed pretreatment drug pyridostigmine also failed to prevent DFP's effects. Atropine was the only drug tested which blocked the attenuation of photoresponse caused by DFP.

4. The fact that atropine blocked the attenuation of photoresponse by DFP suggests that ACh accumulation at receptor sites might be involved in the photoresponse attenuation. However, atropine instead may be preventing access of DFP to its site of action. Various lines of evidence suggest that DFP may act directly on ion channels. The fact that DFP changes neither membrane resistance nor the reversal potential of the photoresponse suggests that its action is associated specifically on ion channels with the photoresponse.

5. The photoresponse of ERP cells is similar to that in rod outer segments, particularly in the release of calcium from intracellular organelles and a subsequent action of the intracellular messenger on plasma membrane ion channels. The results reported here point to the exciting possibility that organophosphates may have a direct effect on retinal photoreceptors, attenuating their photoresponse.

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